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Advances in technology for detection of plant pathogenic microorganisms

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Abstract. DNA diagnostics are developing at a rapid pace being fuelled by the needs of the medical diagnostic and genomics industries. The genomics industry is based on high throughput analysis of genomes in the search for DNA markers linked to valuable traits. The industry requires analysis of thousands of samples and a major criterion is that the cost of analysis must be kept low. The development of high throughput facilities based on robotic workstations, solid state PCR and MALDI-TOF analysis makes this feasible with a cost of less than $1 per sample. In parallel, microarray technology has developed to the stage where it is possible to screen entire genomes for genetic defects instantaneously. This is increasingly being applied in human and veterinary medicine and in other areas such as environmental health, water analysis, certification of transgenic foodstuffs, and in the food industry where instant genotyping or pathogen profiling is a priority. It is inevitable that these technologies will impact on plant pathology diagnostics. Already in Australia, and other parts of the world we are seeing the establishment of commercial high throughput services offering pathogen profiling of soil samples using either DNA probe technology or MALDI-TOF analysis.

Introduction

Plant disease causing microorganisms (bacteria, fungi, viruses) are a major limitation on the productivity of plant production in agricultural ecosystems. A major cost of plant production is the cost of management of plant diseases through the application of fungicides, preventing the spread of disease by quarantine, and by the breeding of resistant varieties. Diseases have the potential to wipe out entire industries with very serious economic consequences. In natural ecosystems diseases can reduce biodiversity of plant species and consequently of animal species which depend on the plants. Plant deaths from disease may also raise the watertable leading to salt degradation of the land.

Diagnostics is a major weapon in the fight against plant-disease causing micro-organisms. The ability to detect the pathogen at an early stage in disease development allows the timely implementation of disease control outbreaks such as the application of fungicides or removal of the infected material. Quarantine is an important component of disease management and, in a physically isolated country like Australia, has been successful in keeping out a number of serious plant diseases. However the increased movement of plant germplasm and soil both within, and between, national borders in recent years has increased the potential for the spread of
plant diseases. Pathogens are carried as contaminants in asymptomatic material such as seeds, or fruit or in products derived from plants. The problems are exacerbated by the increased number of people travelling by air bringing with them food and other material such as wooden ornaments which may harbour pathogens. This means that there is a need for greater vigilance by quarantine services coupled with a need for more effective methods for detection of pathogens.

Traditionally pathogens are detected either by plating samples on microbial growth media, or where appropriate by planting seeds (under quarantine conditions) to see if any diseased plants grow. The problems associated with these are (a) they take some considerable time and resources, and (b) the pathogen may not grow or even cause disease despite being present. It may not be until after the importation has been approved, and a particular set of environmental and/or physiological conditions occurs that the disease manifests itself.

An alternative approach would be to detect the DNA of the pathogen. This detection method is independent of environmental conditions or of the growth stage of the pathogen. In recent years there has been a tremendous increase in the application of molecular biology techniques to the detection of phytopathogenic microorganisms in asymptomatic plant material (Bej and Mahbubani 1992; Bonants, *et al.* 1997; Dunn, *et al.* 1995; Ersek *et al.* 1994; Kappe *et al.* 1996; Navot *et al.*2001; O'Brien 1996; Ristaino *et al.* 1998; Schots *et al.*

1994; Skinner 1992; Varma and Kwon-Chung 1992; Wiglesworth *et al.* 1994). This is based on results showing that such tests are sensitive, rapid and highly specific. There have been numerous reports in the scientific literature on the development of DNA probes, or more commonly PCR tests for detection of phytopathogenic bacteria, fungi and viruses.

PCR Tests

The most common type of molecular test developed is based on the Polymerase Chain Reaction (PCR) (Schots *et al.* 1994). This requires a DNA sequence that is specific to the pathogen. Such sequences exist in all organisms, usually as the sequences between genes where there is no selective pressure either for or against nucleotide substitutions. These sequences can be used to differentiate not only species, but also subspecific groups, eg., pathotypes.

The PCR Process

In the PCR reaction, a specific target DNA sequence 200-1000 base pairs long is amplified several billion times. Amplification starts with heat denaturation of the DNA to separate the strands (usually at approx 92^{0}C), followed by the annealing of short (15-20 bases) oligonucleotide primers to sites flanking the target sequence (temp range 40 to 60^{0}C) (Fig. 1). The primers are extended by a thermostable DNA polymerase (at 72^{0}C) so that we now have four strands of DNA. We initiate a new cycle by heating the DNA to separate the strands, and the process repeats. At each cycle the number of DNA strands doubles and after 35-40 cycles there are enough copies so that we can see the DNA on a gel or by some other means. Each stage in the process (primer annealing, extension and separation of the strands) occurs at a different temperature which is achieved by carrying out the reaction in a thermalcycler which is programmed to go to specific temperatures in a defined sequence.

Usually the products are analysed by electrophoresis on agarose or acrylamide gels although alternative methods for analysis exist. One of these is the Taq Man system developed and marketed by Applied Biosystems. In this process extension of the primers by DNA polymerase generates fluorescence. The amount of fluorescence increases with each cycle. This allows us to follow the product accumulation after each cycle. This is called "real time PCR" and can be used to quantify the amount of template in the starting material.

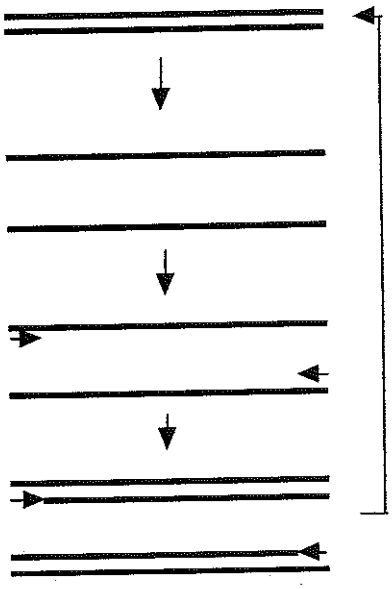

Fig. 1. The PCR process

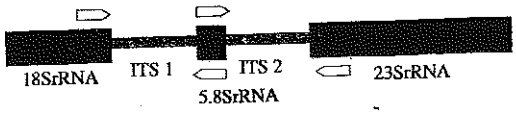

Fig. 2. Organization of the ribosomal RNA (rRNA) genes. The arrows indicate primers used for amplification of the ITS regions.

The majority of PCR diagnostic tests for fungal pathogens focus on the Internal Transcribed Spacers (ITS) of the ribosomal RNA (rRNA) genes (Fig. 2). The reasons for this are threefold: (i) is that the sequences at the ends of the small subunit (SSU) and large subunit (LSU) genes are conserved and so we can use the same primers to amplify the ITS sequences from many different species without first sequencing the genes (Kappe *et al.* 1996; Medlin *et al.* 1988). From the sequence of the amplified ITS regions we can develop species specific primers. (ii) because nucleotide substitutions within the ITS region are phenotypically neutral, the sequences are highly variable and hence a good source of species specific probes. (iii) The rRNA genes are multicopy, being repeated hundreds of times in bacteria and thousands of times in eukaryotic nuclei. The high copy number makes them easy to detect.

An advantage of PCR detection tests is that they are extremely sensitive. A good example of this is the generation of an individual's DNA fingerprint from the saliva left on a cigarette butt. Similarly in plant pathology, a minute fragment of a root, stem or leaf can be enough to carry out analysis. A caveat is that the distribution of the pathogen within the sample may not be uniform, and hence we may fail to detect it.

Application of PCR detection tests in commercial diagnostics

From a research point of view PCR has provided a tremendous boost to the study of plant pathology. It has revolutionised our ability to probe pathogen population structure and to investigate the distribution of the pathogen in natural ecosystems and in plants.

Despite the advantages of PCR tests in terms of sensitivity specificity and rapidity, these tests have not moved out of the research laboratory into commercial application. There are very few commercial diagnostic operations based on PCR. Those that are tend to be government funded institutions which are often not working on full cost recovery, or commercial operations which deal with large numbers of samples (high throughput facilities). The main factor limiting the adoption of PCR detection test by industry is the cost of the process. At the current state of development it is an expensive process which in addition requires highly skilled labour and expensive equipment.

The major cost component of PCR analysis is labour. The reagents used for amplification comprise only a minor cost component by comparison. The processes of extracting DNA setting up PCR reactions, and analysis of the PCR products by gel electrophoresis are time consuming. By replacing some of these steps with less labour intensive procedures the costs per sample can be decreased considerably (Fig. 3).

New developments in PCR diagnostics

Robotic workstations.

The development of automated procedures for extraction of DNA from samples is one approach which has been intensively investigated in recent years as a means of overcoming this limitation on the commercial application of PCR diagnostics. The manipulations involved in DNA extraction are carried out by robotic workstations which handle hundreds of samples simultaneously (Ranjard, *et al.* 2001). They can carry out procedures such as extraction of DNA, set up of PCR reactions, loading of the reactions into the thermalcycler, and loading the PCR amplification products onto a gel or into some alternative form of analysis. Robotic workstations enable high throughput facilities to process in the region of 10,000 samples per day with relatively little labour input. This greatly reduces the cost per sample.

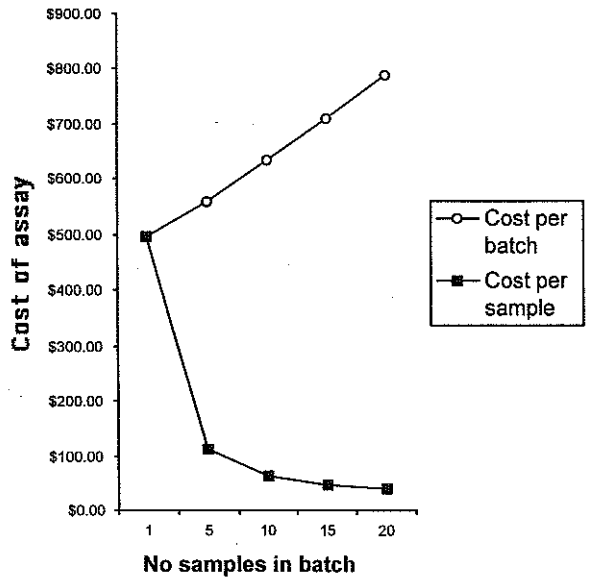

Fig. 3. Effect of sample numbers on costs of PCR analysis. As the number of samples increases the costs per sample decreases.

Mass spectroscopic analysis of DNA.

Another labour intensive component in PCR analysis is analysis of the PCR product by gel electrophoresis. Pouring, loading, running and imaging the gels is time consuming. This problem can be overcome by using real time PCR systems such as the Taq Man system (see above) or more popularly with high throughput facilities, mass spectroscopy.

There are different types of procedures for mass spectroscopic analysis of DNA but the most common and popular is designated Matrix, Assisted Laser Desorption Ionisation-Time of Flight (MALDI-TOF) mass spectroscopy (Bray *et al.* 2001; Monforte and Becker 1997). In this procedure DNA is mixed with a matrix and immobilised on a solid support. The mixture is irradiated with a laser which vaporises the DNA. Disintegration of the DNA is avoided by use of an appropriate matrix which absorbs the initial energy of the laser and transfers it to the DNA. The vaporised DNA under the influence of an electric field then flies down a tube to a detector at the end. The time taken to traverse the tube depends on the *mass* of the DNA and is typically of the order of milliseconds. Batches of 800 samples can be loaded into the MALDI-TOF for analysis. In addition to the speed and automation, another advantage of MALDI-TOF is that very little DNA is required for analysis. This means that only 5-10 cycles of PCR are required instead of the 30-40 cycles normally required.

On site analysis by PCR.

A drawback of the high throughput facilities for PCR diagnostics is that samples have to be transferred to them for analysis. This inevitably means a delay in getting the result. There are situations in which it would be extremely useful to be able to carry out an analysis on the spot eg., testing the nutrient fluid in hydroponics facility for the presence of a pathogen. Advances in microfluidics are making on site detection by PCR in very short time frames a reality. Microfluidics is the miniaturisation of separation and assay techniques such that multiple procedures can be carried out on a single chip made of glass or other polymers (Lemieux *et al.* 1998). Channels etched into the surface of the chip are used to deliver reagents to and from a reaction chamber. Processes such as electrophoresis can also be carried out on a chip and combined with DNA extraction procedures.

Reactions such as PCR amplification can be carried out with the reagents immobilised on the surface of the chip (solid state PCR). Because such small volumes (nL) are required mixing occurs very quickly, as does temperature ramping (shifting between temperatures) due to the very efficient transfer of heat. This means

that PCR amplification can be completed in minutes as compared to the 2-3h required for conventional PCR.

Belgrader *et al.* (1999) have described a microfluidic system for extraction of DNA from bacteria and amplification and analysis of the product within 7 minutes. In another study Hofgartner *et al.* (1999) compared microchip analysis of PCR products from herpes simplex encephalitis virus with the more conventional gel retardation assay. They reported that microchip analysis took less than 110 seconds per sample compared to 18h for the conventional assay and that the level of sensitivity and specificity was comparable to the established method.

Pathogen profiling

Pathogen profiling is the term used for the process of detecting multiple pathogens in a sample. Unlike the cases we have been considering above which involve a test for single pathogens, pathogen profiling enables us to detect multiple pathogens simultaneously. This is made possible through the use of microarrays. Mircoarrays are DNA chips with oligonucleotide sequences immobilised on the surface in a defined pattern (array) such that we know the position of each sequence (Fig. 4). The sequences are derived from genes (usually the rRNA genes) of the organisms we are trying to detect. DNA is extracted from the sample and hybridised to the immobilised sequences on the chip. The extent of hybridisation to each target sequence is determined by labelling the DNA. Using a microarray scanner we can measure the amount of DNA hybridised to each target sequence on the filter. How many pathogens can be detected on a single chip? Microarrays containing 10,000 sequences cm^2 are often used for gaining a profile of gene expression in a single cell, or for genotyping, although much higher density arrays have been described (Lemieux *et al.* 1998). Although this number if certainly greater than would be required for plant pathology it demonstrates the potential of the technique for detecting multiple pathogens. For plant pathology the arrays would be much simpler consisting of perhaps less than one hundred sequences. This makes them cheaper to construct.

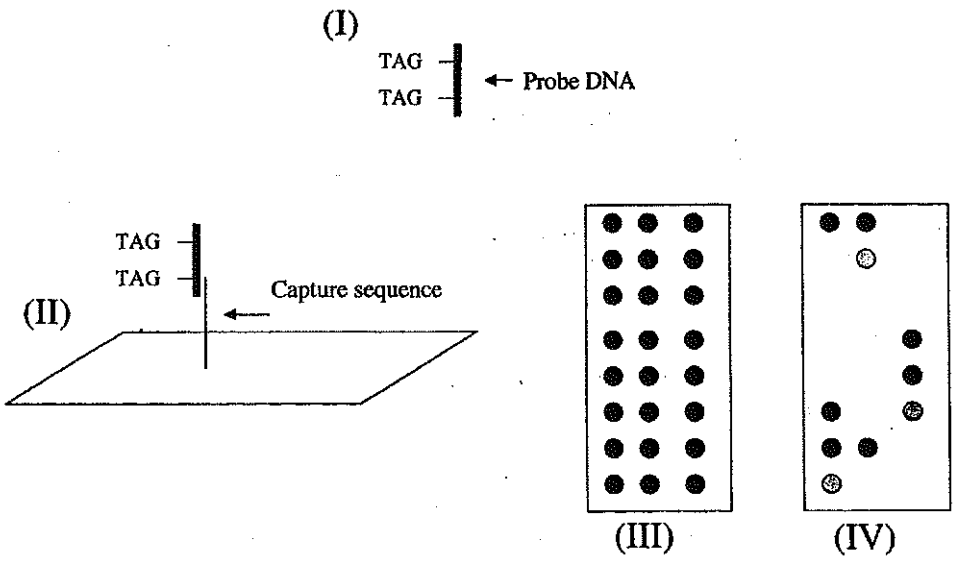

Fig. 4. Detection of pathogens using microarrays. The probe DNA is extracted from the assay sample and labelled with a fluorescent tag (I). The labelled DNA is hybridised to a chip on which the capture sequences are immobilised (II). Usually the chip has multiple capture sequences immobilised in a defined pattern (array) (III). From the amount of fluorescence on each spot of the array we can determine what pathogens are present in the sequence and their amount (IV).

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